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(54) Title: TISSUE COMPOSITIONS USING CULTURED FIBROBLASTS AND KERATINOCYTES AND METHODS OF USE THEREOF

(57) Abstract: Compositions comprising a biodegradable material having a positive charge, fibroblasts, and keratinocytes, methods of forming the compositions, and methods for treating tissue conditions or disorders using the compositions are provided. The preferred biodegradable material having a positive charge is crosslinked polysaccharide having a positive charge. The compositions are particularly useful in treating various skin tissue conditions or disorders.

**TISSUE COMPOSITIONS USING CULTURED FIBROBLASTS  
AND KERATINOCYTES AND METHODS OF USE THEREOF**

**BACKGROUND**

**Technical Field**

This disclosure relates to compositions useful for treating damaged tissue, methods of preparing such compositions, and methods for treating certain tissue  
5 conditions and disorders. In particular, such compositions include a biodegradable material having a positive charge, fibroblasts and keratinocytes.

**Description of Related Art**

Skin is one of the largest and most important organs of the body. It is  
10 composed of two regions, an outer layer of epithelial tissue known as the epidermis and an inner layer of connective tissue known as the dermis. The epidermis is multilayered and is composed mainly of keratinocytes which exist at different stages of differentiation and maturation. Basal cells are the least differentiated of the keratinocytes and occupy the innermost layer of the epidermis, adjacent to the dermis. They are the main cells in  
15 the epidermis undergoing mitosis. Layers of prickly cells are located above the basal cells, followed by a layer of granular cells. The outermost layer of the epidermis consists of dead cells packed with keratin. The dermis is composed largely of connective tissue and is supplied with blood vessels and nerves. The epidermis and dermis are joined together by the basal lamina, which serves to adhere both of these tissues and provides  
20 support for the epidermis. The skin not only plays a key role in temperature regulation of the body and as a sensory organ, but also serves as a protective barrier for the body by preventing infection, and excessive fluid loss.

An individual who has suffered extensive damage or loss of skin caused by wounds, burns, or disease is immediately vulnerable to life-threatening conditions  
25 such as infection and dehydration. To combat these life-threatening conditions, various approaches have focused on transplanting skin to the injured site. One of the most widely

used methods involves harvesting a section of skin from an undamaged site of an individual and transplanting this section of skin to the damaged site of the same individual. This type of transplant is known as an autograft.

While this approach typically results in the successful growth of the autograft at the damaged site, several problems may accompany the use of autografts. In particular, in situations where there is significant skin loss or damage, e.g., as with severe burns, an individual may lack sufficient healthy unburned skin to cover the site of damaged or destroyed skin. Skin grafts can be repeatedly harvested from the same site in an individual, by allowing the harvested site to grow new skin. However, the recovery of the individual is delayed using this procedure, because sufficient time must be allowed for the growth of new skin from the harvested site. In addition, the quality of skin harvested repeatedly from the same site is reduced. Further, the harvesting procedure itself is painful, and results in scarring. Moreover, particularly in individuals suffering from massive burns, the harvested skin may be meshed and stretched to cover the burned area, which results in scarring.

Alternatively, skin can also be harvested from a donor species and transplanted to a recipient of a different species, e.g., donor pig to recipient human, which is referred to as a xenograft. Xenografts are useful, particularly in individuals who have suffered skin loss or damage over a significant area of the body. However, xenografts may undergo rejection by the recipient, and thus, can only serve as a temporary covering for the skin.

Another approach to covering the site of extensive skin loss or damage is to transplant frozen or fresh human cadaveric skin. This type of transplant is referred to as an allograft, and is described, e.g., in Atnip et al., Curr. Prob. Surg. 20: 623-86; Pruitt et al., Arch. Surg. 119:312-22; Hansbrough, In: Boswick J. Ed. The Art and Science of Burn Care. Rockville, Md: Aspen Publ. Inc., 57-63, 1987. In general, allografts are transplants from one member of a species to another member of the same species. While cadaveric skin provides a viable substitute for autografts, particularly when the supply of autograft skin is limited, it suffers from the same disadvantage as xenografts, i.e.,

rejection. In addition, the use of cadaveric allograft skin exposes an individual to potential transmission of various diseases, e.g., AIDS and hepatitis.

Consequently, in view of the aforementioned drawbacks, particular strategies have been employed to develop skin substitutes made of various synthetic and biological materials, particularly collagen and nondegradable and biodegradable polymers. For example, Yannis et al., in U.S. Patent No. 4,060,081, describes a synthetic multi-layered membrane useful as synthetic skin comprising a layer of an insoluble and nondegradable crosslinked collagen-mucopolysaccharide composite and a moisture control transmission layer.

Orgill et al., in U.S. Patent No., 5,716,411, describes methods of regenerating skin utilizing a collagen-glycosaminoglycan matrix having an outer moisture barrier, i.e., silicone, which is subsequently replaced by a cultured epithelial autograft (CEA) sheet.

Bell, in U.S. patent 4,485,097, describes a skin equivalent composed of a hydrated collagen lattice, which is contracted into a living tissue using fibroblasts, and keratinocytes deposited on the contracted lattice.

Mark, in U.S. Patent No. 5,282,859, describes a skin equivalent including an epidermal layer of non-porous collagen and keratinocytes, and a dermal layer of fibroblasts in a porous crosslinked collagen sponge matrix.

Bakker et al., in U.S. Patent No. 5,147,401, describes an artificial skin made up of a top layer of a segmented thermoplastic copolymer and an underlayer of a degradable biocompatible polymer, wherein the top and bottom layers can be provided with epithelial cells and fibroblasts, respectively.

Hansbrough et al., in U.S. Patent No. 5,460,939, describes a synthetic skin composed of living stromal tissue prepared from fibroblasts or other stromal cells cultured on a synthetic or biodegradable framework, and a transitional covering bonded to the framework.

Griffith-Cima et al., in U.S. Patent 5,709,854 describes a method of injecting cell-polymeric solutions into an animal to form tissues made of a polymeric hydrogel containing dispersed cells.

5 Green et al., in U.S. Patent No. 4,304,866 describes a method of producing transplantable sheets of living keratinous tissue by culturing keratinocytes in a culture vessel and enzymatically degrading the sheet of keratinous tissue formed.

While the aforementioned skin substitutes may be useful in providing a viable alternative to natural skin, improved methodologies and different biologic approaches are desirable for replacing damaged or destroyed skin tissue.

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### SUMMARY

In one aspect, a composition is provided which includes a biodegradable material having a positive charge, fibroblasts, and keratinocytes.

15 In another aspect, a composition useful for treating tissue conditions and disorders is provided which includes biodegradable crosslinked polysaccharide beads having a positive charge, and fibroblasts.

In another aspect, a composition useful for treating tissue conditions or disorders is provided which includes biodegradable crosslinked polysaccharide beads having a positive charge, and keratinocytes.

20 In another aspect, a method of preparing a composition useful for treating tissue conditions or disorders is provided which includes a biodegradable material having a positive charge, fibroblasts, and keratinocytes, which includes preparing a layer including the biodegradable material and the fibroblasts by culturing the fibroblasts with the biodegradable material under conditions favoring adhesion of the fibroblasts onto the surface of the biodegradable material sufficient to allow the fibroblasts to grow, and associating another layer including keratinocytes with the layer including the biodegradable material and the fibroblasts.

25

In another aspect, a method of treating tissue conditions or disorders is provided which includes administering to a subject in need thereof an effective amount of

a composition which includes a biodegradable material having a positive charge, fibroblasts, and keratinocytes, sufficient to treat the tissue condition or disorder.

In yet another aspect, a kit for forming a composition useful for treating tissue conditions or disorders is provided which includes a separate portion of each of a) a biodegradable material having a positive charge, b) fibroblasts, and c) keratinocytes.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A-1D are photomicrographs showing aggregation of NIH 3T3 fibroblasts at various cell concentrations to positively charged beads.

Figure 2 is a graph of cell concentration versus concentration of positively charged beads. The graph shows that  $300-400 \times 10^3$  cells/3mg beads/ml yields the largest aggregates and that increasing or decreasing the concentration of beads from a concentration of 3 mg/beads/ml causes a decline in aggregation.

Figure 3 shows the size of cell/bead aggregates formed using positively charged, negatively charged or uncharged beads and various combinations of these beads.

Figure 4A and 4B is a photomicrograph showing the binding of keratinocytes to positively charged beads.

Figures 5A and 5B are photomicrographs showing adhesion of NIH 3T3 fibroblasts to fibronectin in the presence of uncharged, negatively charged or positively charged beads. Figure 5A shows adhesion of cells to positively charged beads but not to uncharged or negatively charged beads when cells were added 4 hours prior to the addition of the beads. Figure 5B shows adhesion of cells to positively charged beads but not to uncharged or negatively charged beads when the beads and cells were added at the same time.

Figures 6A and 6B are photomicrographs showing adhesion of NIH 3T3 fibroblasts to fibronectin in the presence (A) or absence (B) of positively charged beads, when fibroblasts were pre-incubated with fibronectin for 10, 20, and 30 minutes prior to the addition of the beads.

Figures 7A and 7B are photomicrographs showing adhesion of NIH 3T3 fibroblasts to laminin, fibronectin and collagen IV in the presence of positively charged (A) or uncharged (B) beads.

Figures 8A and 8B are photomicrographs showing the adhesion of NIH 3T3 fibroblasts to polylysine, bovine serum albumin-coated or uncoated culture dishes in the presence of positively charged (A) or uncharged (B) beads.

Figure 9 is a photomicrograph showing NIH 3T3 fibroblasts adhered to positively charged beads to form cell/bead aggregates.

#### **DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS**

The present disclosure relates to compositions useful for treating tissue conditions or disorders, methods for preparing the compositions, and methods for treating various tissue conditions or disorders. The aforementioned problems accompanying the use of autografts, xenografts, and cadaveric skin allografts, are addressed herein by providing compositions useful for treating various tissue conditions or disorders, e.g., destroyed or damaged skin tissue resulting from burns, wounds, or disease. The compositions supplement or replace native tissues such as, e.g., skin, and can be grown in relatively large commercially viable quantities.

The compositions described herein include biodegradable material having a positive charge, keratinocytes and fibroblasts. Fibroblasts adhere to and rapidly proliferate on the biodegradable material, thereby providing large quantities of living tissue that can replace damaged or destroyed tissue. The term "tissue" includes skin and various types of connective tissue, e.g., tendon and ligaments. The term "biodegradable" means that the material having the positive charge is substantially degraded by naturally occurring processes in a living organism such as hydrolysis into components that are absorbed in the body. The material having the positive charge may also be biocompatible, i.e., that it does not elicit substantially adverse affects, e.g., rejection or undue irritation, when transplanted to the damaged or destroyed tissue site.

The present compositions are particularly useful in the treatment of patients requiring replacement or supplementation of skin, due to extensive burns, wounds, diseases, etc. Since the compositions can be readily prepared in large quantities from a small number of cells, particularly from the patient's own cells, they are especially useful in cases where the patients lack sufficient healthy skin for autografts. Accordingly, the compositions provide an effective solution to the problem of a shortage of autograft skin, and rejection of xenografts and cadaveric skin allografts.

An advantage of the compositions described herein is that they include living cells such as fibroblasts that are present in normal tissue, e.g., skin tissue. Upon adherence and growth on the biodegradable material, the fibroblasts secrete matrix proteins such as fibronectin and collagen, and cytokines, and thus, the compositions come to approximate the function of naturally occurring tissues, particularly of skin. Generally, most anchorage-dependent cells, i.e., cells that will grow only when attached to an appropriate surface, e.g., fibroblasts, when added to a culture dish adhere to extracellular matrix proteins secreted from the cells or deposited from the serum such as fibronectin, laminin or collagen (see also Example 6). In accordance with the present disclosure, the presence of matrix proteins does not inhibit or prevent the fibroblasts from binding to the biodegradable material having a positive charge as shown below (see Examples 6-9). Accordingly, anchorage-dependant cells are able to adhere and proliferate on the surface of the biodegradable material even in the presence of matrix proteins. Indeed, as shown in Example 9, fibroblasts adhered to positively charged beads interact with an extracellular matrix protein on the bead's surface to survive for a long period of time.

Another advantage of the present compositions is that the material having the positive charge not only provides a scaffold upon which fibroblasts and other cells can adhere and proliferate, but also enhances the migration of cells from the surrounding healthy tissue toward the material when the composition is transplanted to the destroyed or damaged tissue site. Accordingly, the incorporation of fibroblasts into the composition and enhanced migration of cells from the surrounding area into the transplanted composition results in the enhanced formation of viable tissue having increased strength



and durability. In addition, the biodegradable material having a positive charge degrades and is absorbed by the body, thus leaving the resulting tissue formed of cells which is functionally and structurally similar to naturally produced tissue.

5 In one embodiment, the compositions include a biodegradable material having a positive charge, fibroblasts and keratinocytes and are prepared by adding the biodegradable material having a positive charge to a container, e.g., a culture dish, adding fibroblasts and allowing them to grow and proliferate, and then adding keratinocytes. Alternatively, the biodegradable material having a positive charge is added to a container, keratinocytes are then added to the container and allowed to grow, followed by the  
10 addition of fibroblasts. Yet another embodiment of the aforementioned compositions is formed by adding biodegradable material having a positive charge to a container, followed by the addition of a mixture of fibroblasts and keratinocytes, and allowing them to grow.

In one aspect, a composition herein includes a layer including the  
15 biodegradable material having a positive charge and fibroblasts, and another layer including keratinocytes which associates with the layer including the biodegradable material and fibroblasts. The fibroblasts adhere to and proliferate on the surface of the biodegradable material and secrete matrix proteins and cytokines. The layer including the biodegradable material and fibroblasts is the functional equivalent of the dermal layer of  
20 skin. In one embodiment, the layer including keratinocytes also includes a biodegradable material having a positive charge. The keratinocytes adhere to and proliferate on the surface of the biodegradable material. The layer including keratinocytes is the functional equivalent of the epidermal layer of skin.

Association of the layer including the biodegradable material and the  
5 fibroblasts, with the layer including keratinocytes can occur through direct contact, wherein the layer of keratinocytes adheres to the layer including the biodegradable material and fibroblasts. It is also contemplated that association of both layers of the composition can occur indirectly by associating a side of both layers with another layer(s), which can include a natural or synthetic polymer, e.g., collagen,

glycosaminoglycans, and mixtures thereof, or with a membrane made of natural or synthetic biodegradable materials. Accordingly, the composition formed provides a substitute for the structure and function of naturally occurring skin.

Suitable biodegradable materials having a positive charge include, but  
5 are not limited to, natural and synthetic materials such as polymeric materials, wherein the positive charge is naturally present or chemically induced on the material. The biodegradable material having the positive charge may be in the form of a bead, sheet, foam, mesh, and so forth. Examples of natural and synthetic biodegradable polymers include, but are not limited to, polysaccharides and proteins. Preferably, the material  
10 having a positive charge is a polysaccharide, which may be ionically or covalently crosslinked. The polysaccharides contemplated herein may be rendered biodegradable as described below. More preferably, the crosslinked polysaccharide is in the form of a bead. Suitable polysaccharides that can be ionically crosslinked are well known in the art and include alginic acid and pectic acids which complex with particular multivalent  
15 ions such as  $\text{Ca}^{++}$  to provide ionic crosslinking. Preferably, the polysaccharide is covalently crosslinked, and includes polysaccharides such as dextran and modified alginates. More preferably, the covalently crosslinked polysaccharide is crosslinked dextran, which is commercially available, e.g., under the tradename, Sephadex from Pharmacia Corp. (Piscataway, N.J.), which is a bead. Modified alginates may be  
20 prepared as described in PCT WO 93/09176, which is incorporated herein by reference.

A positive charge can be provided on the crosslinked polysaccharide beads by reaction with suitable functional groups, e.g., diethylaminoethyl (DEAE) groups, using techniques that are well known in the art as described, e.g., in Eppley et al., U.S. Patent No. 5,092,883 and Eppley et al., U.S. Patent No. 4,988,358, both of  
25 which are incorporated herein by reference. Crosslinked dextran having DEAE groups is commercially available under the tradename DEAE-Sephadex from Pharmacia Corp. (Piscataway, N.J.), which is a bead.

Examples of other crosslinked polysaccharide beads suitable for use herein which can be rendered biodegradable as described below, include Sepharose and Sephacel beads of Pharmacia Corp. (Piscataway, N.J.). Both types of beads may be provided with the DEAE functional group. The Sepharose beads are derived from  
5 agarose while the Sephacel beads are derived from cellulose.

Techniques for preparing biodegradable crosslinked polysaccharides are well known in the art. For example, a biodegradable crosslinked polysaccharide can be produced by oxidizing the crosslinked polysaccharide, e.g., with periodic acid or a periodate salt, to produce linkages which are unstable under hydrolytic conditions as  
10 described, e.g., in Gruskin et al., U.S. Patent No. 5,502,042, which is incorporated herein by reference. When the crosslinked polysaccharide is rendered biodegradable by oxidation, preferably, the positive charge provided on the crosslinked polysaccharide is induced on the polysaccharide prior to oxidation. The rate of degradation of the crosslinked polysaccharide can be controlled by varying the concentration of oxidizing  
15 agent and the reaction time for oxidation, as described, e.g., in Gruskin et al., *supra*, to allow sufficient time for the fibroblasts to adhere to and proliferate on the crosslinked polysaccharide to produce a composition, and for the composition to become a viable living tissue of the body upon transplantation to a destroyed or damaged tissue site. Preferably, the oxidized crosslinked polysaccharide is oxidized crosslinked dextran  
0 wherein the positive charge on the cross-linked dextran is provided by diethylaminoethyl groups.

Fibroblasts for use herein may be harvested from any suitable organ of any human or animal, preferably from the same type of tissue to be cultured. More preferably the fibroblasts are harvested from skin. Recent research as described, e.g., in Hansbrough  
5 et al., J. Burn Care Rehab. 13: 519-29, 1992; and Cuono et al., Lancet 1: 1123-4, 1986, has shown that human fibroblasts are relatively non-antigenic, and thus do not provoke immediate rejection upon transplantation to an allogenic host. Accordingly, the fibroblasts may be obtained from an animal's own tissues, i.e., autologous fibroblasts, or from an animal of the same or of a foreign species, i.e., heterologous fibroblasts.

Neonatal or fetal fibroblasts may also be utilized. Heterologous fibroblasts may be genetically altered by introducing a gene expressing a protein that inhibits or prevents rejection of the heterologous cells upon transplantation to a recipient species, e.g., a complement inhibitory factor. In addition, the fibroblasts may be obtained from established cell culture lines.

Methods of isolating and disaggregating fibroblasts from suitable organs or tissues are well known to those skilled in the art, as described, e.g., in Hansbrough et al., in U.S. Patent 5,460,939; and Freshney, Culture of Animal Cells. A Manual of Basic Technique, 2nd Ed., A. R. Liss, New York, pp. 107-126, and 137-168, 1987, both of which are incorporated herein by reference. Typically, a skin sample, e.g., human neonatal foreskin, can be mechanically or enzymatically dissociated into individual cells. For example, mechanical separation can be achieved using a homogenizer, grinder, blender, etc. Enzymatic separation can be achieved using a variety of digestive enzymes e.g., trypsin, chymotrypsin, collagenase, elastase, etc. Once the tissue is disaggregated into individual cells, fibroblasts may be separated from other cells in the tissue by conventional methods, such as fluorescence-activating cell sorting, clonal selection of specific types of cells, etc.

Keratinocytes for use herein may be obtained from the human or animal having the damaged tissue, i.e., autologous keratinocytes, or they may be obtained from a donor of the same or different species, i.e., heterologous keratinocytes. Methods of dissociating and isolating keratinocytes are well known to those skilled in the art. For example, to prepare dissociated keratinocytes, the epidermis can be enzymatically, or mechanically separated from the dermis of the skin, followed by separation of the epidermis into small sections or individual keratinocytes. To prevent or inhibit rejection of heterologous keratinocytes, the keratinocytes may be genetically altered by introducing a gene expressing a protein which inhibits or prevents rejection of the cell by the recipient, e.g., a gene expressing a complement inhibitory factor.

In addition to the fibroblasts and keratinocytes, other cells, particularly cells of connective tissue, e.g., skin, may be incorporated into the compositions. Such

cells include, but are not limited to, endothelial cells, adipocytes, pericytes, macrophages, monocytes, leukocytes, plasma cells, mast cells, reticulocytes and combinations thereof. These cells may also be obtained from suitable tissue, and preferably from the tissue being cultured. Methods of isolating these cells are well-known to those skilled in the art as described, e.g., in Freshney, *supra*, pp. 107-126, and 137-168.

The compositions described herein may further include a biocompatible biodegradable natural or synthetic polymer. As mentioned previously, the term "biocompatible" means that the natural or synthetic polymer does not elicit substantially adverse affects, e.g., rejection or undue irritation, when transplanted to the damaged or destroyed tissue site. These polymers are also degraded by natural processes of the organism, e.g., by hydrolysis and then are absorbed in the body. Biocompatible biodegradable synthetic polymers may include, but are not limited to, polylactic acid, polyglycolic acid, polyorthoester, polycarbonates such as trimethylene carbonate, polycaprolactone and combinations thereof as described, e.g., in Kennedy, U.S. Patent No. 5,102,983, which is incorporated herein by reference. Suitable biocompatible biodegradable synthetic polymers also include lysine ethyl ester diisocyanate derivatives as described, e.g., in Bennett et al., U.S. Patent No. 5,578,662, which is incorporated herein by reference. Biodegradable natural polymers include, but are not limited to, albumin, synthetic polyamino acids, and polysaccharides such as alginate, heparin, etc. The biodegradable polymer may be coated with a second substance which enhances cell attachment to the polymer, e.g., fibronectin, glycosaminoglycans (heparin sulfate, chondroitin sulfate, keratin sulfate), basement membrane components, polylysine, etc.

The composition may further include an active agent. For example, matrix proteins, growth factors, angiogenesis promoting factors, cytokines, antimicrobials, anti-inflammatories, and combinations thereof, can be incorporated into or pre-coated on the biodegradable material having the positive charge, or provided in conjunction with the composition, or incorporated into or pre-coated on the biodegradable polymer. Suitable matrix proteins include, but are not limited to, fibronectin, laminin, glycosaminoglycans, type IV collagen, type V collagen, hyaluronic acid, polylysine and combinations thereof.

Growth factors may include, but are not limited to, epidermal growth factor, keratinocyte growth factor, insulin-like growth factors I and II, growth hormone, transforming growth factor- $\beta$  and combinations thereof. Examples of angiogenesis promoting factors include, but are not limited to, basic fibroblast growth factor, vascular endothelial cell growth factor, platelet derived growth factor, angiogenin, angiotropin, heparin sulfate, etc., and combinations thereof. Suitable antimicrobials include, but are not limited to, antibacterial agents, such as antibiotics, antifungal and antiprotozoal agents, as described, e.g., in Remington's Pharmaceutical Sciences, ed. Gennaro, A.R., 17th Ed., Mack Publishing Company, PA, pp. 1158-1233, 1985, which is incorporated herein by reference. Suitable anti-inflammatories include, but are not limited to, dexamethasone, cortisone, hydrocortisone, hydrocortisone acetate, beta methasone, etc.

In one embodiment, a composition useful for treating tissue conditions and disorders may also include either fibroblasts or keratinocytes, and biodegradable crosslinked polysaccharide beads having a positive charge. Preferably, the crosslinked polysaccharide is crosslinked dextran, and preferably the positive charge on the crosslinked polysaccharide is provided by diethylaminoethyl groups. It is also preferred that the biodegradable crosslinked polysaccharide beads are oxidized, as described, e.g., in Gruskin et al., *supra*. More preferably, the oxidized crosslinked polysaccharide is crosslinked dextran, wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

Another embodiment provides for a method of preparing a composition including a biodegradable material having a positive charge, fibroblasts, and keratinocytes. This method includes preparing a layer including the biodegradable material having a positive charge and the fibroblasts, by culturing the fibroblasts with the biodegradable material having a positive charge under conditions favoring adhesion of the fibroblasts onto the surface of the material sufficient to allow the fibroblasts to grow as described below, and associating a layer of keratinocytes with the layer including the biodegradable material and fibroblasts. Preferably the biodegradable material is a biodegradable crosslinked polysaccharide as described above, and more preferably the

crosslinked polysaccharide is crosslinked dextran. The positive charge on the crosslinked polysaccharide is preferably provided by diethylaminoethyl groups. The biodegradable crosslinked polysaccharide is preferably oxidized, as described above. The most preferred oxidized crosslinked polysaccharide is oxidized crosslinked dextran, wherein  
5 the positive charge on the oxidized crosslinked dextran is provided by diethylaminoethyl groups.

The layer including the biodegradable material having a positive charge and fibroblasts is generally prepared by culturing *in vitro* the biodegradable material with the fibroblasts which are harvested and isolated as described above, under conditions  
10 sufficient to allow the fibroblasts to adhere and proliferate onto the surface of the biodegradable material, prior to transplantation of the composition. The type of biodegradable material having a positive charge, proportion of the biodegradable material to number of cells to be cultured, amount of time, and conditions under which the fibroblasts are cultured *in vitro* may be ascertained by measuring cell adherence, amount  
15 of proliferation, and the percentage of successful transplanted compositions. Typically, fibroblasts and crosslinked polysaccharide beads as the biodegradable material having the positive charge are cultured in a serum-free medium, e.g., Dulbecco's Modified Eagle's Medium (DMEM) in 10% total bovine serum/FBS for about 1 to about 2 hours to allow the cells to adhere to the surface of the material. The adhered cells are then grown on the  
20 material for about 1-2 weeks.

Alternatively, the fibroblasts may be cultured *in vivo*, by first allowing the cells to adhere to the biodegradable material *in vitro*. Whether the cells are cultured *in vitro* prior to transplantation, or *in vivo* generally depends on the amount of cells available for culturing, and the amount of destroyed or diseased tissue to be replaced. In addition  
25 to culturing the fibroblasts on the biodegradable material having the positive charge, the fibroblasts may be cultured on natural or biosynthetic biocompatible biodegradable polymers which have been described above. The layer including keratinocytes may be prepared by harvesting skin and isolating the keratinocytes as described above.

Dissociated keratinocytes as described above may then be cultured *in vitro* on culture media, e.g., keratinocyte serum-free medium (SFM; Life Technologies, Inc., Gaithersburg, MD) to produce CEA (cultured epithelial autograft) sheets made of several layers of different types of differentiated keratinocytes. Methods of preparing CEA sheets are well known in the art and described, e.g., in U.S. Patent 5,716,411, and U.S. Patent 4,304,866, both of which are herein incorporated by reference. It is also contemplated that the layer including keratinocytes can be prepared *in vitro* or *in vivo* by distributing a suspension of the dissociated keratinocytes onto the layer of the biodegradable material and fibroblasts, and allowing the keratinocytes to proliferate and differentiate into the functional equivalent of the epidermal layer.

In a manner similar to fibroblasts, the layer including keratinocytes can also include a biodegradable material having a positive charge. Dissociated keratinocytes can be cultured with such biodegradable material by allowing the keratinocytes to adhere to and proliferate on the surface of such material. Attachment of the keratinocytes to the biodegradable material having the positive charge preferably proceeds *in vitro*. Proliferation of the keratinocytes and formation of the layer including keratinocytes and biodegradable material having a positive charge can occur *in vitro* or *in vivo*. Preferably, such biodegradable material is a crosslinked polysaccharide having a positive charge, which is a bead. The crosslinked polysaccharide is preferably crosslinked dextran, and the positive charge on the crosslinked polysaccharide is preferably provided by diethylaminoethyl groups. The biodegradable crosslinked polysaccharide is preferably oxidized, as described above. Most preferably, the oxidized crosslinked polysaccharide is crosslinked dextran, wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

The layer including keratinocytes, alone or including the biodegradable material having a positive charge, is associated with the layer including the biodegradable material and fibroblasts, usually by direct contact of the two layers. For example, contact can occur, e.g., by distributing a suspension of dissociated keratinocytes alone or adhered to the material having the positive charge, using a spatula or a needleless syringe, or by



transferring confluent CEA sheets or keratinocytes grown on the biodegradable material having a positive charge onto the layer including fibroblasts by fastening a gauze-type of material to the surface of the CEA sheet or to the layer of keratinocytes grown on the biodegradable material using surgical clips.

5 In a preferred embodiment, the fibroblasts are added to a culture dish, and allowed to adhere to the dish and proliferate. The biodegradable material having a positive charge, e.g., beads, is then added to the culture dish. The fibroblasts lift off the surface of the culture dish and adhere to and proliferate on the beads. Subsequently, keratinocytes are added to the dish which also adhere to and proliferate on the beads. The  
10 composition formed may then be lifted out the culture dish and placed directly on an excised wound, with the outer surface of the layer including fibroblasts in direct contact with the excised wound, and the layer including keratinocytes exposed directly to the air.

In an alternative embodiment, the biocompatible biodegradable polymers mentioned previously are first added to a culture dish followed by the addition of  
15 keratinocytes which adhere and proliferate on the surface of the polymer. Subsequently, the biodegradable material having a positive charge, e.g., in the form of beads, is added to the culture dish, followed by the addition of fibroblasts which adhere to and proliferate on the beads. The composition formed can then be peeled off the surface of the dish and placed in contact with the excised wound so that the polymer surface of the layer  
0 including keratinocytes is directly exposed to the air, and the layer including fibroblasts is in direct contact with the excised wound.

As stated above, the association of the two layers of the tissue graft may also occur indirectly, by contacting one side of each layer of the composition with a layer(s) or membrane made of natural or synthetic polymers.

5 In another aspect, the compositions described above can be utilized to treat a human or animal having various tissue conditions, particularly skin conditions, e.g., wounds and burns, or skin disorders, e.g., melanomas, necrotizing subcutaneous infection caused by bacteria, etc., by augmenting or replacing the damaged or destroyed tissue with the composition. Treatment is effected by administering an effective amount of the

composition described herein, sufficient to treat the tissue condition or disorder. The effective amount of the composition depends on the nature of the tissue condition or disorder and the extent of damaged or destroyed tissue. The effective amount can be readily determined by a surgeon by examining the destroyed or damaged tissue site in need of replacement.

Prior to transplanting the composition to the desired site, the complete site of damaged or destroyed tissue, e.g., a wound or a burn, may be debrided or surgically excised to permit proper healing of the site upon grafting of the composition. Various *in vitro* and *in vivo* approaches to placing and growing the compositions are contemplated for treating a human or animal suffering from a tissue condition or disorder. For example, the entire composition may be prepared *in vitro*, shaped to fit in the excised site, and transplanted in the excised tissue site with the side of the layer including keratinocytes which is non-associated with the layer including fibroblasts and biodegradable material having a positive charge, exposed to the air. In another approach, the layer including the biodegradable material and fibroblasts can be grown *in vitro* and transplanted to the excised site, or grown in the excised tissue site itself. Distribution of the keratinocytes, on the layer including the fibroblasts and the biodegradable material having a positive charge can be performed at the time the layer including fibroblasts and biodegradable material is formed *in vitro*, or any time after the fibroblasts proliferate and form a confluent layer in the excised tissue site. Prior to distributing the keratinocytes on the transplanted layer including the biodegradable material and the fibroblast, a temporary biodegradable membrane or barrier may be placed on top of the transplanted layer, to prevent infection and excessive loss of fluid, until the keratinocytes form a confluent layer. Transplantation of either layer alone can be achieved by any convenient method. For example, either layer can be applied to the excised tissue site using a spatula or needleless syringe, or by fastening a gauze-type of material to either layer using surgical clips, or shaped from a mold to fit into the excised tissue site.

A confluent layer including keratinocytes can be secured to a confluent layer including fibroblasts and biodegradable material having a positive charge which has

been transplanted into an excised tissue site with sutures or surgical staples, which allows the layer including keratinocytes to firmly adhere to the layer including the keratinocytes and biodegradable material having a positive charge. In addition, the layer including fibroblasts and biodegradable material grown *in vitro* or to confluence *in vivo* can also be  
5 secured to the adjacent tissue area through the use of sutures or surgical staples.

In a further embodiment, a kit is provided which includes a separate portion of each of a biodegradable material having a positive charge, fibroblasts, and keratinocytes. The kit may also include a biocompatible biodegradable polymer as described above. The kit may further include one or more containers, e.g., a container for  
10 biodegradable material in the form of beads, culture dishes, for culturing the cells and for holding or molding the final compositions which are to be subsequently transplanted to the damaged tissue site. The biodegradable material having the positive charge can be any natural or synthetic polymer, as described above, and is preferably crosslinked polysaccharide in the form of a bead. Preferably, the crosslinked polysaccharide is  
15 crosslinked dextran. The positive charge on the crosslinked polysaccharide is preferably provided by diethylaminoethyl groups. It is also preferred that the biodegradable crosslinked polysaccharide is oxidized as described above, and most preferred that the oxidized crosslinked polysaccharide is crosslinked dextran, wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups. The cells supplied in  
20 the kit are preferably frozen, and thawed prior to use. The kit may be assembled by any convenient method. For example, when the biodegradable material having a positive charge supplied in the kit is crosslinked polysaccharide beads, fibroblasts may be added to a culture dish, and then cultured as described above. The aforementioned beads are then added to the culture dish, wherein the fibroblasts lift off the surface of the culture  
25 dish and adhere to and proliferate on the beads. Keratinocytes which are dissociated can be suspended in a suitable medium and distributed onto the layer including fibroblasts and biodegradable material having a positive charge, e.g., using a needleless syringe. Alternatively, the keratinocytes may also be grown on the aforementioned beads or grown into CEA sheets and then transferred onto the layer including fibroblasts and

biodegradable material, e.g., by fastening a gauze-type of material to the surface of the CEA sheet or to the layer of keratinocytes grown on the biodegradable material using surgical clips.

The following examples are included for purposes of illustrating certain embodiments and are not intended to limit the scope of this disclosure.

### **EXAMPLE 1**

#### **Adhesion of Mouse Fibroblasts and Macrophages to Positively Charged Crosslinked Polysaccharide Beads**

The adhesiveness of mouse fibroblasts and macrophages to a material having a positive charge was examined by incubating the cells with DEAE Sephadex A-25 beads. NIH 3T3 mouse fibroblasts and IC-21 mouse macrophages were radiolabeled by incubating  $3\text{-}5 \times 10^5$  cells and 0.25-0.5mCi of Translabel Methionine  $^{35}\text{S}$  (ICN Biomedicals, Inc., Costa Mesa, CA) in flasks overnight. Subsequently the cells were washed with Dulbecco's Modified Eagle's Medium (DMEM) in 10% FBS to remove unincorporated  $^{35}\text{S}$ -methionine three times, and trypsinized with DMEM containing 10% FBS. Subsequently, the cells were washed with DMEM containing no serum and suspended in the same medium. Beads (Pharmacia Corp., uncharged: Sephadex G-25, negatively charged: CM Sepharose A-25, or positively charged: DEAE Sephadex A-25) at concentrations of 1 mg/ml or 5 mg/ml were washed twice with DMEM containing no serum and resuspended in the same medium.

The radiolabeled cells were then incubated with the beads in a flask for one hour. The beads were then washed, and the cells which adhered to the beads were lysed. The counts per minute (CPM) of the lysed cells were then measured. The results of these studies are shown in Tables 1 and 2.

**Table 1**  
**Percentage of bound cells to different types of beads.**

Type of cells	Uncharged beads 5 mg/ml	Negatively charged beads 5 mg/ml	Positively charged beads 5mg/ml
NIH 3T3 cells	0.0%	2.3%	30.0-45.7%
IC-21 cells	0.0%	0.% Microscope	28.8%-66.0%

-Microscope means that no numbers were obtained but the wells containing cells and beads were examined under the microscope.

-0% indicates that no cells bound to beads.

**Table 2**  
**Comparing the adhesion of cells to 1 mg/ml vs  
 5 mg/ml of positively charged beads.**

Type of cells	Positively charged beads 1 mg/ml	Positively charged beads 5mg/ml
NIH 3T3 cells	17.0%	45.7%
IC-21 cells	5.3%	28.8%

The data shown in Table 1 is the percentage of cells (measured in CPM) (Beckman Counter, Fullerton, CA) adhered to beads out of the total cells added. The data clearly show that fibroblasts and macrophages adhere more to positively charged beads than to uncharged or negatively charged beads. Table 2 shows that there is an increase in cell binding as the concentration of beads increases.

## **EXAMPLE 2**

### **Aggregation of Fibroblasts and Macrophages to Positively Charged Beads at Different Concentrations of Cells and Crosslinked Polysaccharide Beads**

5

Adherent NIH 3T3 mouse fibroblasts and IC-21 macrophages were removed and washed with Versene-EDTA solution. The EDTA was neutralized with DMEM containing 10% FBS. Cells were washed twice with DMEM containing no serum and suspended in the same medium. Beads (Pharmacia Corp., uncharged: Sephadex G-25, negatively charged: CM Sepharose A-25, or positively charged: DEAE Sephadex A-25) were washed with DMEM containing no serum and then suspended in the same medium. DMEM (0.25 ml) containing 3 mg/ml of the above washed beads was added to a 24 wells culture dish. Subsequently, the following concentration of cells were added to each well: 50-100-250-500 x 10<sup>3</sup> and 1-2 x 10<sup>6</sup> cells. The beads and cells in each culture dish were incubated for 1 hour at 37°C, and aggregation was examined. The results of the aggregation of cells on positively charged beads at various concentrations of the cells are shown in the photomicrographs, Figures 1A-1D. These photomicrographs show that 250-300 x 10<sup>3</sup> cells/3 mg positively charged beads/ml gave the most aggregation of cells and beads.

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Subsequently, 350 x 10<sup>3</sup> cells (prepared as described above) were added to a 24 wells culture dish, followed by the addition of different concentrations of positively charged beads (0.25, 0.5, 1, and 2-5 mg/ml). The beads and cells were incubated for 1 hour at 37°C, and aggregation was examined. A graph of cell concentration vs. bead concentration (Figure 2) shows that increasing or decreasing the concentration of beads from a bead concentration of 3mg/ml caused a decline in aggregation.

25

Equal number of beads (uncharged negatively or positively charged beads) were mixed and seeded on a 24 wells culture dish (Falcon 3047). Subsequently, 400x10<sup>3</sup> cells were added to each well. The following combination of beads were added to each well: 1.5 mg/ml each of uncharged and negatively charged beads, 1.5 mg/ml each of

uncharged and positively charged beads, 1.5 mg/ml each of positively and negatively charged beads, 3 mg/ml of uncharged beads, 3 mg/ml of negatively charged beads, and 3 mg/ml of positively charged beads. The beads and cells were incubated for 1 hour in 37°C and aggregation was examined.

5                   The results (Figure 3) demonstrated that the negatively and positively charged bead combination formed aggregations similar to aggregations formed by cells and positively charged beads.

### 10                   EXAMPLE 3

#### Proliferation of Fibroblasts on Positively Charged Beads

NIH 3T3 mouse fibroblasts at concentrations of 1,000, 2000, 5,000, and 10,000 cells/tube were allowed to adhere to and proliferate on beads (1mg/ml or 5mg/ml, Pharmacia Corp., positively charged: DEAE Sephadex A-25, uncharged: Sephadex G-25) in DMEM in 10% FBS for five days in 15 ml tubes at 37°C. Subsequently, a thymidine incorporation assay was performed as follows. <sup>3</sup>H-thymidine (2μCi) was added to each tube containing the cell/bead aggregates or beads alone and incubated for 5 hours. The media was then removed, and the cells and the beads were washed with 5 ml of PBS three times. Cold trichloroacetic acid (TCA, 1 ml) was added to each tube and incubated on ice for ten minutes. The TCA was removed, and the process with TCA was repeated two more times. The cells and the beads were washed with distilled water three times and 500μl of 0.3N NaOH was added to each tube for 30 minutes. The mixture was transferred to scintillation vials and the CPM was measured using a beta counter (Beckman Counter, Fullerton, CA).

25                   The results of the proliferation assay indicated that fibroblasts proliferated on positively charged beads (DEAE Sephadex A-25) but not on uncharged beads (Sephadex G-25).

#### **EXAMPLE 4**

##### **Adhesion of Keratinocytes to Positively Charged Beads**

The adhesion of human keratinocytes to uncharged, positively or negatively charged beads was examined. NIH 3T3 fibroblasts (used as a positive control) and human keratinocytes ( $5 \times 10^4$  cells) were washed with DMEM three times, removed with Versene-EDTA, neutralized with DMEM containing 10% FBS, washed again with serum-free DMEM one and resuspended in the same medium. Beads (Pharmacia Corp., positively charged: DEAE Sephadex A-25, uncharged: Sephadex G-25, and negatively charged: CM Sepharose A-25) were washed twice with serum-free DMEM twice and resuspended in the same medium. The cells and beads were allowed to interact in the culture dish for two hours. The adhesion of the cells to the beads was examined under a microscope. As shown in Figures 4A and 4B the NIH 3T3 cells and human keratinocytes bound to the positively charged beads, but not to negatively charged or uncharged beads.

#### **EXAMPLE 5**

##### **Proliferation of Keratinocytes on Positively Charged Beads**

Keratinocytes were added to 6 well culture dishes at cell concentrations of substitute cells in the 5,000, 10,000 or 200,000 cells, and incubated at 37°C for a few days until the cells reached confluence. Positively charged beads (DEAE Sephadex A-25) at 1 mg/ml and 5 mg/ml were added to the wells. The culture dishes were examined every day to determine if the layers of cells were lifted off the culture dish. The floating cell/bead aggregates were then collected and allowed to grow for a few



days. A proliferation assay was then performed using  $^3\text{H}$  thymidine. The results are shown in Table 3.

Table 3

Number of Initial Cells Added to the Beads	Proliferation/CPM
No Cells	1517
5,000 cells	10736
10,000 cells	7542

The data from the proliferation assay indicated that keratinocytes adhered to and proliferated on positively charged beads.

#### EXAMPLE 6

##### Adhesion of Fibroblasts and Macrophages to Beads in the Presence of Extracellular Matrix Molecules

The following experiments were performed to determine the interaction between fibroblasts and macrophages, and different types of beads in the presence of different extracellular matrix molecules (substrates) including laminin, fibronectin, collagen IV and polylysine.

Two plates of 24 wells (Falcon 3047) were coated with 10  $\mu\text{g/ml}$  of laminin, collagen IV, fibronectin or 40  $\mu\text{g/ml}$  of polylysine in phosphate buffered saline (PBS) for 4 hours at 37°C. The plates were washed three times with PBS and blocked with 10-20  $\mu\text{g/ml}$  bovine serum albumin (BSA) overnight at 37°C. The plates were then washed three times with a serum-free medium (DMEM).

##### Experiment A:

Macrophages IC-21 or NIH 3T3 fibroblasts were removed with a Versene-EDTA solution, neutralized with DMEM containing 10% FBS, washed twice with a serum-free medium (DMEM) and resuspended in the same medium. Macrophages IC-21 ( $200 \times 10^3$  cells/well) and NIH 3T3 fibroblasts ( $125 \times 10^3$  (cells/ well) were added to

the above plates and allowed to adhere for 4 hours at 37°C. Subsequently, non-adherent cells were washed away with DMEM.

5               Beads (3 mg/ml, Pharmacia Corp., negatively charged: CM Sepharose 25, positively-charged: DEAE A-25, and uncharged: Sephadex G-25) were washed twice with DMEM and then added to the wells containing the cells. The plates were left in 37°C for 2 hours.

10              Prior to the addition of the beads, both macrophages IC-21 and fibroblasts NIH 3T3 did not bind to laminin-coated wells, however, both type of cells adhered and spread on collagen IV, fibronectin and polylysine. Two hours after the addition of the beads, both negatively charged and uncharged beads did not bind to cells that were seeded on collagen IV, fibronectin and poly-lysine or cells seeded on uncoated/unblocked wells. However, positively charged beads adhered to cells that were seeded on collagen IV, fibronectin and polylysine or cells seeded on uncoated/unblocked wells. Figure 5A shows the adhesion of fibroblasts to positively charged beads but not to uncharged or negatively charged beads in the presence of fibronectin, 4 hours after the beads were added to the cells.

15              These experiments indicate that the binding of cells to collagen IV and fibronectin did not affect the binding of positively charged beads to these cells. The binding of cells to these two substrates also did not alter the inability of negatively charged or uncharged beads from binding to cells.

#### Experiment B:

25              Macrophages IC-21 or NIH 3T3 fibroblasts, culture dishes and beads were prepared as described above. The cells and beads were added at the same time to the coated culture dishes.

              In the presence of negatively charged or uncharged beads, both types of cells adhered and spread on collagen IV, fibronectin and polylysine or on uncoated/

unblocked wells. Both type of cells aggregated with positively charged beads and did not adhere to any of the substrates for the duration of the experiment (2 hours).

These data indicate that when cells are given the choice between collagen IV and positively charged beads or between fibronectin and positively charged beads, they adhere to the positively charged beads. Figure 5B shows the adhesion of fibroblasts to positively charged, but not to negatively charged or uncharged beads in the presence of fibronectin, when the beads and fibroblasts were added at the same time.

#### EXAMPLE 7

##### Adhesion of Fibroblasts to Positively Charged Beads of Extracellular Matrix Molecules at Various Time Intervals

The following experiment was performed to determine the interaction between fibroblasts and different types of beads, when fibroblasts were incubated with different extracellular matrix molecules (substrates) including laminin, fibronectin, collagen IV, and polylysine at various time intervals, prior to the addition of the beads. Two plates of 24 wells (Falcon 3047) were coated with 10  $\mu\text{g/ml}$  of collagen IV, fibronectin and 40  $\mu\text{g/ml}$  polylysine in PBS for 8 hours at 37°C, washed three times with PBS, blocked with 20 mg/ml BSA in PBS overnight at 37°C, and then washed three times with a serum-free medium (DMEM). NIH 3T3 fibroblasts were removed with a Versene-EDTA solution, neutralized with DMEM containing 10% FBS, washed twice with a serum-free medium (DMEM) and resuspended in the same medium. The fibroblasts were added to the plates at a final concentration of  $125 \times 10^3$  cells/ml and allowed to adhere for: 15, 30 or 45 minutes. Non-adherent cells were washed away with a serum-free medium (DMEM).

Positively charged beads (DEAE Sephadex A-25) were washed twice with a serum-free medium (DMEM) and resuspended in the same medium and added to the wells at a concentration of 3 mg/ml.

Then for each extracellular matrix molecule the following wells were prepared: 2 wells with positively charged beads and 2 wells without beads for each

time-point: 15, 30, and 45 minutes. The culture dishes were then incubated at 37°C for 1 hour, and washed three times with PBS. Subsequently, the cells were fixed with 3% paraformaldehyde at pH 7.2 and stained.

As shown in Figures 6A and 6B, positively charged beads did not have any effect on fibroblasts that were added and spread on fibronectin for longer than 15 minutes, i.e., the beads adhered to the cells. The adhesion of the beads to the cells in the first 10 minutes was weak since the beads were washed away during the wash. The above experiment suggests that the preference of cells to bind to positively charged beads over matrix molecules occurs in a period of less than 15 minutes.

#### **EXAMPLE 8**

##### **Reaction of Cells from Extracellular Molecules Upon Exposure to Positively Charged Beads**

The following experiment was performed to determine the interaction between cells and different types of beads in the presence of different extracellular matrix molecules including laminin, fibronectin, collagen IV and polylysine. The length of this experiment was 12 hours instead of 2 hours with previous experiments.

A 24 well culture dish was coated (Costar 3524) with either 20  $\mu\text{g/ml}$  of laminin, 10  $\mu\text{g/ml}$  of Collagen IV, h-fibronectin (10  $\mu\text{g/ml}$ ) or 40  $\mu\text{g/ml}$  of polylysine in PBS for three hours at 37°C. The wells were then blocked with 50 mg/ml BSA in PBS for 3 hours at 37°C, and washed three times with a serum-free medium (DMEM).

NIH 3T3 fibroblasts, IC-21 macrophages or HUVEC endothelial cells were removed with Versene-EDTA and neutralized with DMEM containing 10% FBS. NIH 3T3 fibroblasts ( $150 \times 10^3$  cells/ml), IC-21 macrophages ( $150 \times 10^3$  cells/ml) and HUVEC endothelial cells ( $250 \times 10^3$  cells/ml) were added to the coated wells and allowed to adhere overnight in their serum-containing medium.

The beads (3 mg/ml, Pharmacia Corp., positively charged: DEAE Sephadex A-25, and uncharged: Sephadex G-25) were washed with DMEM containing 10% FBS twice and then resuspended in the same medium and added to the wells.

For each cell type, the following wells were prepared: a set of 4 wells per substrate (laminin, collagen IV, h-fibronectin), a set of 4 wells blocked with BSA, and a set of 4 wells unblocked or coated with BSA. Out of each set of 4 wells, the following beads were added: 2 wells for positively charged beads, 1 well for uncharged beads, and 1 well without any beads. The culture dishes were left at 37°C for 12 hours. Non-adherent cells were removed by washing three times with PBS. Adherent cells and beads were fixed with 3% paraformaldehyde at pH 7.2 and stained.

On all substrates and on uncoated/unblocked wells and without beads NIH 3T3 fibroblasts bound and spread well.

On laminin, collagen IV and fibronectin in the presence of positively charged beads, the beads bound to the fibroblasts, and fibroblasts bound to the substrates (Figure 7A). However, more cells were bound to the beads than cells bound and spread on the substrates. On laminin, collagen IV, fibronectin in the presence of uncharged beads, the beads did not bind to the fibroblasts (Figure 7B). The cells were well spread on the different substrates similar to the wells where no beads were present.

On polylysine or BSA in the presence of positively charged beads, fibroblasts were not bound to the substrate, but instead were bound to the beads (Figure 8A). The cell-bead aggregates were floating in the medium. On polylysine or BSA in the presence of uncharged beads, fibroblasts were well-bound to the beads and spread similar to cells in wells that had no beads (Figure 8B). HUVEC cells behaved similarly to NIH 3T3 fibroblasts on all substrates.

IC-21 macrophages were detached from all substrates in the presence of positively charged beads and were bound and spread on the same substrates in the presence of uncharged beads or no beads.

These results showed that cells that were well-bound and spread to different extracellular matrix molecules for at least 12 hours before beads were added, retracted from the substrates and adhered instead to the positively charged beads. The presence of positively charged beads appeared to turn off the ability of cells to adhere to matrix molecules.

Cells that did not spread well on matrix molecules such as macrophages were more affected by the presence of the positively charged beads than the more well spread cells such as fibroblasts and endothelial cells, i.e., there were more macrophages bound to the beads and less bound to the substrates.

5                These observations suggest that positively charged beads affect the cells in two ways, they turn off their interaction with the extracellular matrix molecule and turn on their interaction with the beads themselves.

#### **EXAMPLE 9**

##### **Survival of Fibroblasts and Macrophages on Positively Charged Beads in the Absence of Binding to any Extracellular Matrix Protein**

10                Most adherent cells die if they are prevented from adhering to a culture dish or extracellular matrix proteins. In Examples 1, 2 and 4, the binding of fibroblasts, macrophages and keratinocytes to positively charged beads or the binding of fibroblasts and macrophages to various extracellular matrix proteins (see Examples 6-8) was examined for a short period of time. In this study, fibroblasts and endothelial cells were allowed to adhere to positively charged beads (DEAE Sephadex A-25) and the formed cell/bead aggregates were maintained in suspension for a week to determine if the cells survive by binding to positively charged beads.

20                NIH 3T3 fibroblasts (400,000 cells/well) and HUVEC endothelial cells (80,000 cells/well) were incubated with different types of DMEM-washed beads (Pharmacia Corp., uncharged: Sephadex G-25, negatively charged: CM Sepharose A-25, positively charged: DEAE Sephadex A-25) in 6 well culture dishes containing complete medium for one week. Cell/bead aggregates formed only with the positively charged beads. The cell/bead aggregates were transferred to a 100 mm culture dish, and washed with DMEM three times and trypsinized. The cells that were dissociated from the aggregates were left in the cultures dishes for one day, to allow the cells to adhere to the beads.

Dissociated cells from the cell/bead aggregates that were floating in the dish were viable cells since after trypsinization occurred the cells adhered and spread on the culture dish.

5 In a second study, NIH 3T3 fibroblasts ( $400 \times 10^3$ ), and HUVEC  
endothelial cells ( $80 \times 10^4$ ) were incubated with different types of DMEM-washed beads  
(3 mg/ml) as described above and allowed to adhere for 2 hours. Cells that did not adhere  
to the beads were adhered and spread on the culture dishes. Cell-bead aggregates were  
transferred to a 15 ml conical tube and were left in a complete medium for 1 week.  
Subsequently, the cell-bead aggregates were transferred to culture dishes and cells on the  
10 beads were examined.

Since the cell-bead aggregates were left in a 15ml conical tube, the cells  
did not interact with any culture dish or extracellular matrix protein except soluble  
fibronectin in the serum. The cells trypsinized from the cell-positively charged bead  
aggregates were viable after 1 week. The cells were spread on the positively charged  
15 beads and some were adhered but remained rounded.

These data indicate that the surface of positively charged beads presents an  
adherent surface to which cells remain viable as if they were bound to a culture dish or  
extracellular matrix protein. These data also indicate that cells adhered to positively  
charged beads interact with an extracellular matrix protein on the beads' surface to  
20 survive for a long time. These data are in agreement with studies that show that cells  
added to a culture dish adhere to extracellular matrix proteins secreted from the cells or  
deposited from the serum such as fibronectin, laminin or collagen IV.

#### **EXAMPLE 10**

##### **Adhesion of Mouse Fibroblasts and Macrophages to Oxidized Positively Charged Crosslinked Polysaccharide Beads**

The adhesiveness of mouse fibroblasts and macrophages to a  
biodegradable material having a positive charge is examined by incubating the cells with  
30 oxidized DEAE Sephadex A- 25 beads. NIH 3T3 mouse fibroblasts and IC-21 mouse

macrophages are radiolabeled by incubating  $3\text{-}5 \times 10^5$  cells and 0.25-0.5mCi of Translabel Methionine  $^{35}\text{S}$  (ICN Biomedicals, Inc., Costa Mesa, CA) in flasks overnight.

Subsequently the cells are washed with Dulbecco's Modified Eagle's Medium (DMEM) in 10% FBS to remove unincorporated  $^{35}\text{S}$ -methionine three times, and trypsinized with

5 DMEM containing 10% FBS. Subsequently, the cells are washed with DMEM containing no serum and suspended in the same medium. Beads (Pharmacia Corp., uncharged: Sephadex G-25, negatively charged: CM Sepharose A-25, or positively charged: DEAE Sephadex A-25, at concentrations of 1 mg/ml or 5 mg/ml) which are made oxidized by the procedure disclosed in Gruskin et al., *supra*, are washed twice with  
10 DMEM containing no serum and resuspended in the same medium.

The radiolabeled cells are then incubated with the beads in a flask for one hour. The beads are then washed, and the cells which adhere to the beads are lysed. The counts per minute (CPM) of the lysed cells are then measured.

The results of these studies show that fibroblasts and macrophages adhere  
15 more to positively charged beads than to uncharged or negatively charged beads. In addition, there is an increase in cell binding as the concentration of beads increases.

In another study, NIH 3T3 fibroblasts were removed with Versene-EDTA and neutralized with a serum-containing medium. The cells were then washed with a serum-free DMEM twice and resuspended in the same medium. Positively charged beads  
20 (Pharmacia, DEAE 25 Sephadex) rendered biodegradable by the procedure described by Gruskin et al., *supra*, were also washed with DMEM twice and resuspended in the same medium. Positively charged beads (1 mg/ml) were added to each well and an increasing number of cells were added to the wells ( $50\text{-}100\text{-}200$  and  $400 \times 10^3$ ). The biodegradable positively charged beads formed suspended cell-bead aggregates as shown in Figure 9.



**EXAMPLE 11****Aggregation of Fibroblasts and Macrophages to Oxidized  
Positively Charged Beads at Different Concentrations of Cells  
and Crosslinked Polysaccharide Beads**

Adherent NIH 3T3 mouse fibroblasts and IC-21 macrophages are removed and washed with Versene-EDTA solution. The EDTA is neutralized with DMEM containing 10% FBS. Cells are then washed twice with DMEM containing no serum and suspended in the same medium. Beads (Pharmacia Corp., uncharged: Sephadex G-25, negatively charged: CM Sepharose A-25, or positively charged: DEAE Sephadex A-25) are made biodegradable by the procedure disclosed in Gruskin et al., *supra*, and washed with DMEM containing no serum and then suspended in the same medium. DMEM (0.25 ml) containing 3 mg/ml of the above washed beads is then added to 24 wells culture dish. Subsequently, the following concentration of cells are added to each well: 50-100-250-500 x 10<sup>3</sup> and 1-2 x 10<sup>6</sup> cells. The beads and cells in each culture dish are incubated for 1 hour at 37°C, and aggregation is examined. The results of the aggregation of cells on positively charged beads at various concentrations of the cells would tend to show that 250-300 x 10<sup>3</sup> cells/3 mg positively charged beads/ml yields the most aggregation of cells and beads.

Subsequently, 350 x 10<sup>3</sup> cells (prepared as described above) are added to 24 wells culture dish, followed by the addition of different concentrations of positively charged beads (0.25, 0.5, 1, and 2-5 mg/ml). The beads and cells are incubated for 1 hour at 37°C, and aggregation is examined. Accordingly, increasing or decreasing the concentration of beads or cells causes a decline in aggregation.

Equal number of beads (uncharged negatively or positively charged beads) are mixed and seeded on 24 wells culture dish (Falcon 3047). Subsequently, 400x10<sup>3</sup> cells are added to each well.

The following combination of beads are added to each well: 1.5 mg/ml each of uncharged and negatively charged beads, 1.5 mg/ml each of uncharged and positively charged beads, 1.5 mg/ml each of positively and negatively charged beads, 3

mg/ml of uncharged beads, 3 mg/ml of negatively charged beads, and 3 mg/ml of positively charged beads.

The beads and cells are incubated for 1 hour in 37°C and aggregation is examined.

Accordingly, the negatively and positively charged bead combination form aggregations similar to aggregations formed by cells and positively charged beads.

### **EXAMPLE 12**

#### **Proliferation of Fibroblasts on Oxidized Positively Charged Beads**

NIH 3T3 mouse fibroblasts at concentrations of 1,000, 2000, 5,000, and 10,000 cells/tube are allowed to adhere to and proliferate on beads (1mg/ml or 5mg/ml, Pharmacia Corp., positively charged: DEAE Sephadex A-25, uncharged: Sephadex G-25), made biodegradable as described previously, in DMEM in 10% FBS for five days in 15 ml tubes at 37°C. Subsequently, a thymidine incorporation assay is performed as follows. <sup>3</sup>H-thymidine (2μCi) is added to each tube containing the cell/bead aggregates or beads alone and incubated for 5 hours. The media is then removed, and the cells and the beads are washed with 5 ml of PBS three times. Cold trichloroacetic acid (TCA, 1 ml) is added to each tube and incubated on ice for ten minutes. The TCA is removed, and the process with TCA is repeated two more times. The cells and the beads are washed with distilled water three times and 500μl of 0.3N NaOH is added to each tube for 30 minutes. The mixture is transferred to scintillation vials and the CPM is measured using a beta counter (Beckman Counter, Fullerton, CA).

The results of the proliferation assay would indicate that fibroblasts proliferate on positively charged beads (DEAE Sephadex A-25) but not on uncharged beads (Sephadex G-25).

**EXAMPLE 13****Adhesion of Keratinocytes to Oxidized Positively Charged Beads**

The adhesion of human keratinocytes to uncharged, positively or negatively charged beads is examined. NIH 3T3 fibroblasts (used as a positive control) and human keratinocytes (5-10-20x10<sup>4</sup> cells) are washed with DMEM three times, removed with Versene-EDTA, neutralized with DMEM containing 10% FBS, washed again with serum-free DMEM one and resuspended in the same medium. Beads (Pharmacia Corp., positively charged: DEAE Sephadex A-25, uncharged: Sephadex G-25, and negatively charged: CM Sepharose A-25) are rendered biodegradable as described previously, and are washed twice with serum-free DMEM twice and resuspended in the same medium. The cells and beads are allowed to interact in the culture dish for two hours. The adhesion of the cells to the beads is examined under a microscope. Accordingly, human keratinocytes bind to the biodegradable beads, but not to negatively charged or uncharged beads.

**EXAMPLE 14****Proliferation of Keratinocytes on Oxidized Positively Charged Beads**

Keratinocytes are added to 6 well culture dishes at cell concentrations of substitute cells in the 5,000, 10,000 or 200,000 cells, and incubated at 37°C for a few days until the cells reached confluence. Positively charged beads (DEAE Sephadex A-25) which are rendered biodegradable as described previously, at 1 mg/ml and 5 mg/ml are added to the wells. The culture dishes are examined every day to determine if the layers of cells are lifted off the culture dish. The floating cell/bead aggregates are then collected and allowed to grow for a few days. A proliferation assay is then performed using <sup>3</sup>H thymidine. Accordingly, keratinocytes adhere to and proliferate on positively charged beads

### **EXAMPLE 15**

#### **Adhesion of Fibroblasts and Macrophages to Oxidized Beads in the Presence of Extracellular Matrix Molecules**

5 The following experiments are performed to determine the interaction between fibroblasts and macrophages, and different types of biodegradable beads in the presence of different extracellular matrix molecules (substrates) including laminin, fibronectin, collagen IV and polylysine.

10 Two plates of 24 wells (Falcon 3047) are coated with 10  $\mu\text{g/ml}$  of laminin, collagen IV, fibronectin or 40  $\mu\text{g/ml}$  of polylysine in phosphate buffered saline (PBS) for 4 hours at 37°C. The plates are washed three times with PBS and blocked with 10-20  $\mu\text{g/ml}$  bovine serum albumin (BSA) overnight at 37°C. The plates are then washed three times with a serum-free medium (DMEM).

#### **Experiment A:**

15 Macrophages IC-21 or NIH 3T3 fibroblasts are removed with a Versene-EDTA solution, neutralized with DMEM containing 10% FBS, washed twice with a serum-free medium (DMEM) and resuspended in the same medium. Macrophages IC-21 ( $200 \times 10^3$  cells/well) and NIH 3T3 fibroblasts ( $125 \times 10^3$  (cells/well) are then added to the above plates and allowed to adhere for 4 hours at 37°C. Subsequently, 20 non-adherent cells are washed away with DMEM.

Beads (3 mg/ml, Pharmacia Corp., negatively charged: CM Sepharose 25, positively-charged: DEAE A-25, and uncharged: Sephadex G-25) are rendered biodegradable as described previously, are washed twice with DMEM and then added to the wells containing the cells. The plates are left in 37°C for 2 hours.

25 Prior to the addition of the beads, both macrophages IC-21 and fibroblasts NIH 3T3 do not bind to laminin-coated wells, however, both type of cells adhere and spread on collagen IV, fibronectin and polylysine. Two hours after the addition of the beads, both negatively charged and uncharged beads do not bind to cells that are seeded on collagen IV, fibronectin and poly-lysine or cells seeded on uncoated/unblocked wells.

However, positively charged beads adhere to cells that are seeded on collagen IV, fibronectin and polylysine or cells seeded on uncoated/unblocked wells adhere to positively charged beads but not to uncharged or negatively charged beads in the presence of fibronectin, 4 hours after the beads are added to the cells.

Accordingly, the binding of cells to collagen IV and fibronectin does not affect the binding of positively charged beads to these cells. The binding of cells to these two substrates also does not alter the inability of negatively charged or uncharged beads from binding to cells.

#### Experiment B:

Macrophages IC-21 or NIH 3T3 fibroblasts, culture dishes and beads are prepared as described above. The cells and beads are added at the same time to the coated culture dishes.

In the presence of negatively charged or uncharged beads, both types of cells adhere and spread on collagen IV, fibronectin and polylysine or on uncoated/unblocked wells. Both type of cells aggregate with positively charged beads and do not adhere to any of the substrates for the duration of the experiment (2 hours).

Accordingly, when cells are given the choice between collagen IV and positively charged beads or between fibronectin and positively charged beads, they will adhere to the positively charged beads.-Fibroblasts adhere to positively charged, but not to negatively charged or uncharged beads in the presence of fibronectin, when the beads and fibroblasts are added at the same time.

#### EXAMPLE 16

##### Adhesion of Fibroblasts to Oxidized Positively Charged Beads of Extracellular Matrix Molecules at Various Time Intervals

The following experiment is performed to determine the interaction between fibroblasts and different types of biodegradable beads, when fibroblasts are incubated with different extracellular matrix molecules (substrates) including laminin,

fibronectin, collagen IV, and polylysine at various time intervals, prior to the addition of the beads. Two plates of 24 wells (Falcon 3047) are coated with 10  $\mu\text{g/ml}$  of collagen IV, fibronectin and 40  $\mu\text{g/ml}$  polylysine in PBS for 8 hours at 37°C, washed three times with PBS, blocked with 20 mg/ml BSA in PBS overnight at 37°C, and then washed three times  
5 with a serum-free medium (DMEM). NIH 3T3 fibroblasts are removed with a Versene-EDTA solution, neutralized with DMEM containing 10% FBS, washed twice with a serum-free medium (DMEM) and resuspended in the same medium. The fibroblasts are then added to the plates at a final concentration of  $125 \times 10^3$  cells/ml and allowed to adhere for: 15, 30 or 45 minutes. Non-adherent cells are washed away with a  
10 serum-free medium (DMEM).

Positively charged beads (DEAE Sephadex A-25) which are rendered biodegradable as described above, are washed twice with a serum-free medium (DMEM) and resuspended in the same medium and added to the wells at a concentration of 3  
mg/ml.

15 Then for each extracellular matrix molecule the following wells are prepared: 2 wells with positively charged beads and 2 wells without beads for each time-point: 15, 30, and 45 minutes. The culture dishes are then incubated at 37°C for 1 hour, and washed three times with PBS. Subsequently, the cells are fixed with 3% paraformaldehyde at pH 7.2 and stained.

20 Accordingly, positively charged beads do not have any effect on fibroblasts that are added and spread on fibronectin for longer than 15 minutes, i.e., the beads adhere to the cells. The adhesion of the beads to the cells in the first 10 minutes is weak since the beads are washed away during the wash. The above experiment would suggest that the preference of cells to bind to positively charged beads over matrix  
25 molecules occurs in a period of less than 15 minutes.

### EXAMPLE 17

#### Reaction of Cells from Extracellular Molecules Upon Exposure to Oxidized Positively Charged Beads

5           The following experiment is performed to determine the interaction between cells and different types of beads in the presence of different extracellular matrix molecules including laminin, fibronectin, collagen IV and polylysine. The length of this experiment is 12 hours instead of 2 hours with previous experiments.

10           A 24 wells culture dish is coated (Costar 3524) with either 20  $\mu\text{g/ml}$  of laminin, 10  $\mu\text{g/ml}$  of Collagen IV, h-fibronectin (10 $\mu\text{g/ml}$ ) or 40  $\mu\text{g/ml}$  of polylysine in PBS for three hours at 37°C. The wells are then blocked with 50 mg/ml BSA in PBS for 3 hours at 37°C, and washed three times with a serum-free medium (DMEM).

15           NIH 3T3 fibroblasts, IC-21 macrophages or HUVEC endothelial cells are removed with Versene-EDTA and neutralized with DMEM containing 10% FBS. NIH 3T3 fibroblasts (150 x 10<sup>3</sup> cells/ml), IC-21 macrophages (150 x 10<sup>3</sup> cells/ml) and HUVEC endothelial cells (250 x 10<sup>3</sup> cells/ml) are added to the coated wells and allowed to adhere overnight in their serum-containing medium.

20           The beads (3 mg/ml, Pharmacia Corp., positively charged: DEAE Sephadex A-25, and uncharged: Sephadex G-25) are made biodegradable as described above and are washed with DMEM containing 10% FBS twice and then resuspended in the same medium and added to the wells.

25           For each cell type, the following wells are prepared: a set of 4 wells per substrate (laminin, collagen IV, h-fibronectin), a set of 4 wells blocked with BSA, and a set of 4 wells unblocked or coated with BSA. Out of each set of 4 wells, the following beads are added: 2 wells for positively charged beads, 1 well for uncharged beads, and 1 well without any beads. The culture dishes are left at 37°C for 12 hours. Non-adherent cells are removed by washing three times with PBS. Adherent cells and beads are then fixed with 3% paraformaldehyde at pH 7.2 and stained.

30           On all substrates and on uncoated/unblocked wells and without beads NIH 3T3 fibroblasts bind and spread well.

On laminin, collagen IV and fibronectin in the presence of positively charged beads, the beads bind to the fibroblasts, and fibroblasts bind to the substrates. However, more cells bind to the beads than cells which are bound and spread on the substrates. On laminin, collagen IV, fibronectin in the presence of uncharged beads, the beads will not bind to the fibroblasts. The cells are well spread on the different substrates similar to the wells where no beads are present.

On polylysine or BSA in the presence of positively charged beads, fibroblasts will not bind to the substrate, but instead will bind to the beads. The cell-bead aggregates are floating in the medium. On polylysine or BSA in the presence of uncharged beads, fibroblasts bind nicely to the beads and spread similar to cells in wells that had no beads. HUVEC cells behave similarly to NIH 3T3 fibroblasts on all substrates.

IC-21 macrophages are detached from all substrates in the presence of positively charged beads and are bound and spread on the same substrates in the presence of uncharged beads or no beads.

Accordingly, cells that bind nicely and spread to different extracellular matrix molecules for at least 12 hours before beads are added, retract from the substrates and adhere instead to the positively charged beads. The presence of positively charged beads appears to turn off the ability of cells to adhere to matrix molecules.

Cells that do not spread well on matrix molecules such as macrophages are more affected by the presence of the positively charged beads than the more well spread cells such as fibroblasts and endothelial cells, i.e., more macrophages will bind to the beads and less will bind to the substrates.

These observations would suggest that positively charged beads affect the cells in two ways: they turn off their interaction with the extracellular matrix molecule and turn on their interaction with the beads themselves.



**EXAMPLE 18****Survival of Fibroblasts and Macrophages on Oxidized  
Positively Charged Beads in the Absence of Binding  
to any Extracellular Matrix Protein**

5

Most adherent cells die if they are prevented from adhering to a culture dish or extracellular matrix proteins. In Examples 10, 12 and 14, the binding of fibroblasts, macrophages and keratinocytes to biodegradable positively charged beads or the binding of fibroblasts and macrophages to various extracellular matrix proteins (see Examples 15-17) is examined for a short period of time. In this study, fibroblasts and endothelial cells are allowed to adhere to positively charged beads (DEAE Sephadex A-25) which are rendered biodegradable as described above, and the formed cell/bead aggregates are maintained in suspension for a week to determine if the cells survive by binding to positively charged beads.

15

NIH 3T3 fibroblasts (400,000 cells/well) and HUVEC endothelial cells (80,000 cells/well) are incubated with different types of DMEM-washed beads (Pharmacia Corp., uncharged: Sephadex G-25, negatively charged: CM Sepharose A-25, positively charged: DEAE Sephadex A-25) which are rendered biodegradable in a 6 wells culture dish containing complete medium for one week. Cell/bead aggregates form only with the positively charged beads. The cell/bead aggregates are then transferred to a 100 mm culture dish, and washed with DMEM three times and trypsinized. The cells that are dissociated from the aggregates are left in the cultures dishes for one day, to allow the cells to adhere to the beads.

20

25

Dissociated cells from the cell/bead aggregates that are floating in the dish are viable cells since after trypsinization occurs the cells adhere and spread on the culture dish.

30

In a second study, NIH 3T3 fibroblasts ( $400 \times 10^3$ ), and HUVEC endothelial cells ( $80 \times 10^4$ ) are incubated with different types of DMEM-washed biodegradable beads (3 mg/ml) as described above and allowed to adhere for 2 hours. Cells that do not adhere to the beads are adhered and spread on the culture dishes. Cell-

bead aggregates are transferred to a 15 ml conical tube and are left in a complete medium for 1 week. Subsequently, the cell-bead aggregates are transferred to culture dishes and cells on the beads are examined.

5 Since the cell-bead aggregates are left in a 15ml conical tube, the cells do not interact with any culture dish or extracellular matrix protein except soluble fibronectin in the serum. The cells typsinized from the cell-positively charged bead aggregates are viable after 1 week. The cells are spread on the positively charged beads and some adhere but remain rounded.

10 These results would indicate that the surface of positively charged beads presents an adherent surface to which cells remain viable as if they are bound to a culture dish or extracellular matrix protein. These data would also indicate that cells which adhere to positively charged beads interact with an extracellular matrix protein on the beads surface to survive for a long time. These data are in agreement with studies that show that cells added to a culture dish adhere to extracellular matrix proteins secreted from the cells or deposited from the serum such as fibronectin, laminin or collagen IV.

15 It will be understood that various modifications may be made to the aspects and embodiments disclosed herein. For example, the compositions have been described as containing layers of materials, e.g., the biodegradable material, fibroblasts, keratinocytes, and biocompatible biodegradable polymers. It is contemplated that the positions of the layers may be interchanged, i.e., various combinations of the compositions herein may be prepared, e.g., in a culture dish, by varying the order of the addition to the culture dish of, a layer including fibroblasts and the biodegradable material having the positive charge and a layer including keratinocytes, each component of both layers, i.e., cells and biodegradable material, and the biocompatible biodegradable polymer to either or both of both layers. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended hereto.

**WE CLAIM:**

1. A composition useful for treating tissue conditions and disorders comprising a biodegradable material having a positive charge, fibroblasts, and  
5 keratinocytes.

2. A composition according to claim 1 having a layer including the biodegradable material having the positive charge and fibroblasts; and another layer including the keratinocytes which associates with the layer including the biodegradable  
10 material and the fibroblasts.

3. A composition according to claim 2 wherein the layer including the keratinocytes further includes a biodegradable material having a positive charge.

4. A composition according to claim 1 wherein the biodegradable material having a positive charge is in the form of a sheet, foam, bead, or combinations thereof.  
15

5. A composition according to claim 3 wherein the biodegradable material having a positive charge is a crosslinked polysaccharide.  
20

6. A composition according to claim 5 wherein the crosslinked polysaccharide is crosslinked dextran.

7. A composition according to claim 5 wherein the positive charge on the crosslinked polysaccharide is provided by diethylaminoethyl groups.  
25

8. A composition according to claim 5 wherein the biodegradable crosslinked polysaccharide is oxidized.

9. A composition according to claim 8 wherein the oxidized crosslinked polysaccharide is crosslinked dextran, and wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

5                   10. A composition according to claim 5 wherein the crosslinked polysaccharide is a bead.

11. A composition according to claim 1 further including a biocompatible biodegradable polymer.

10

12. A composition according to claim 11 wherein the biocompatible biodegradable polymer is selected from the group consisting of polylactic acid, polyglycolic acid, polyorthoester, polycarbonate, polycaprolactone and combinations thereof.

15

13. A composition according to claim 1 further including cells selected from the group consisting of endothelial cells, adipocytes, pericytes, macrophages, monocytes, leukocytes, plasma cells, mast cells, reticulocytes and combinations thereof.

20

14. A composition according to claim 1 wherein the biodegradable material having a positive charge is pre-coated with an active agent selected from the group consisting of a matrix protein, growth factor, angiogenesis promoting factor, cytokine, antimicrobial and combinations thereof.

25

15. A composition according to claim 14 wherein the active agent is a matrix protein selected from the group consisting of fibronectin, laminin, glycosaminoglycans, type IV collagen, type V collagen, hyaluronic acid, polylysine and combinations thereof.

16. A composition according to claim 14 wherein the active agent is a growth factor selected from the group consisting of epidermal growth factor, keratinocyte growth factor, insulin-like growth factor I, insulin-like growth factor II, growth hormone, transforming growth factor  $\beta$  and combinations thereof.

5

17. A composition according to claim 14 wherein the active agent is an angiogenesis promoting factor selected from the group consisting of basic fibroblast growth factor, vascular endothelial cell growth factor, platelet derived growth factor, angiogenin, angiotropin, heparin sulfate and combinations thereof.

10

18. A composition according to claim 2 wherein the layer including the biodegradable material having the positive charge and the fibroblasts further includes cells selected from the group consisting of endothelial cells, adipocytes, pericytes, macrophages, monocytes, leukocytes, plasma cells, mast cells, reticulocytes and combinations thereof.

15

19. A composition useful for treating tissue conditions or disorders comprising a biodegradable crosslinked polysaccharide having a positive charge and fibroblasts.

20

20. A composition according to claim 19 wherein the crosslinked polysaccharide is crosslinked dextran.

21. A composition according to claim 19 wherein the positive charge on the crosslinked polysaccharide is provided by diethylaminoethyl groups.

25

22. A composition according to claim 19 wherein the biodegradable crosslinked polysaccharide is oxidized.

23. A composition according to claim 22 wherein the oxidized crosslinked polysaccharide is crosslinked dextran, and wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

5                   24. A composition comprising a biodegradable crosslinked polysaccharide having a positive charge and keratinocytes.

25. A composition according to claim 24 wherein the crosslinked polysaccharide is crosslinked dextran.

10

26. A composition according to claim 24 wherein the positive charge on the crosslinked polysaccharide is provided by diethylaminoethyl groups.

15

27. A composition according to claim 24 wherein the biodegradable crosslinked polysaccharide is oxidized.

28. A composition according to claim 27 wherein the oxidized crosslinked polysaccharide is crosslinked dextran, and wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

20

29. A method of preparing a composition including biodegradable material having a positive charge, fibroblasts, and keratinocytes which comprises:

preparing a layer including the biodegradable material and the fibroblasts by culturing the fibroblasts with the biodegradable material under conditions favoring adhesion of the fibroblasts onto the surface of the material sufficient to allow the fibroblasts to grow; and associating a layer including keratinocytes with the layer including the biodegradable material and the fibroblasts.

25

30. A method of preparing a composition according to claim 29 wherein the biodegradable material having a positive charge is a crosslinked polysaccharide.

5 31. A method of preparing a composition according to claim 30 wherein the crosslinked polysaccharide is crosslinked dextran.

32. A method of preparing a composition according to claim 30 wherein the positive charge on the biodegradable crosslinked polysaccharide is provided by diethylaminoethyl groups.

10

33. A method of preparing a composition according to claim 30 wherein the biodegradable crosslinked polysaccharide is oxidized.

15 34. A method of preparing a composition according to claim 33 wherein the oxidized crosslinked polysaccharide is crosslinked dextran, and wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

20 35. A method of preparing a composition according to claim 29 wherein the step of preparing the layer including the biodegradable material and fibroblasts, further includes culturing cells selected from the group consisting of endothelial cells, adipocytes, pericytes, macrophages, monocytes, leukocytes, plasma cells, mast cells, reticulocytes and combinations thereof, with the biodegradable material.

25 36. A method of preparing a composition according to claim 29 wherein the biodegradable material having a positive charge is pre-coated with an active agent selected from the group consisting of a matrix protein, growth factor, cytokine, angiogenesis promoting factor, antimicrobial and combinations thereof, prior to the step of preparing the layer including the biodegradable crosslinked polysaccharide beads and the fibroblasts.

37. A method of preparing a composition according to claim 29 wherein the step of preparing the layer including the biodegradable material having a positive charge and the fibroblasts further includes culturing the fibroblasts with a biocompatible biodegradable polymer.

5

38. A method of preparing a composition according to claim 37 wherein the biocompatible biodegradable polymer is selected from the group consisting of polylactic acid, polyglycolic acid, polycarbonate, polyorthoester, polycaprolactone and combinations thereof.

10

39. A method according to claim 29 wherein the keratinocytes are cultured with a biodegradable material having a positive charge, under conditions favoring adhesion of the keratinocytes onto the surface of the biodegradable material sufficient to allow the keratinocytes to grow, prior to the step of associating the layer including keratinocytes with the layer including the biodegradable material and the fibroblasts.

15

40. A method of treating a tissue condition or disorder in a patient comprising administering to the patient in need thereof an effective amount of a composition which includes a biodegradable material having a positive charge, fibroblasts, and keratinocytes, sufficient to treat the skin condition.

20

41. A method of treating a tissue condition or disorder according to claim 40 wherein the biodegradable material having the positive charge is a crosslinked polysaccharide.

25

42. A method of treating a tissue condition or disorder according to claim 41 wherein the crosslinked polysaccharide is crosslinked dextran.



43. A method of treating a tissue condition or disorder according to claim 41 wherein the positive charge on the crosslinked polysaccharide is provided by diethylaminoethyl groups.

5                   44. A method of treating a tissue condition or disorder according to claim 41 wherein the biodegradable crosslinked polysaccharide is oxidized.

                  45. A method of treating a tissue condition or disorder according to claim 44 wherein the oxidized crosslinked polysaccharide is crosslinked dextran, and wherein  
10 the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

                  46. A kit comprising  
                  a separate portion of each of a) a biodegradable material having a positive  
charge, b) fibroblasts, and c) keratinocytes.

15                   47. A kit according to claim 46 further comprising a biocompatible biodegradable polymer.

                  48. A kit according to claim 47 wherein the biocompatible biodegradable  
20 polymer is selected from the group consisting of polylactic acid, polyglycolic acid, polycarbonate, polyorthoester, polycaprolactone and combinations thereof.

                  49. A kit according to claim 46 wherein the biodegradable material having  
a positive charge is a crosslinked polysaccharide.

25                   50. A kit according to claim 49 wherein the crosslinked polysaccharide is crosslinked dextran.

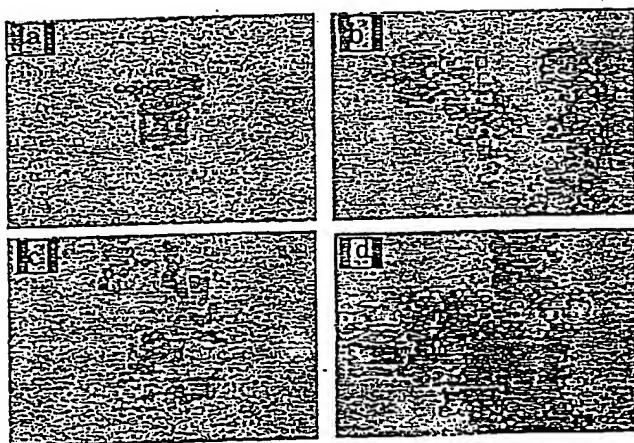
51. A kit according to claim 49 wherein the positive charge on the biodegradable crosslinked polysaccharide is provided by diethylaminoethyl groups.

5 52. A kit according to claim 49 wherein the biodegradable crosslinked polysaccharide is oxidized.

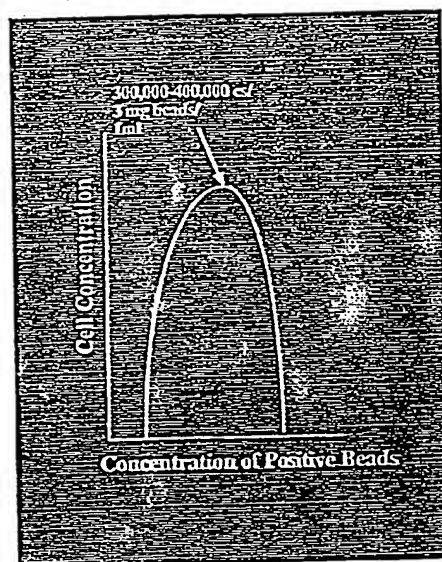
53 A kit according to claim 52 wherein the oxidized crosslinked polysaccharide is crosslinked dextran, and wherein the positive charge on the crosslinked dextran is provided by diethylaminoethyl groups.

10

**FIGURE 1**

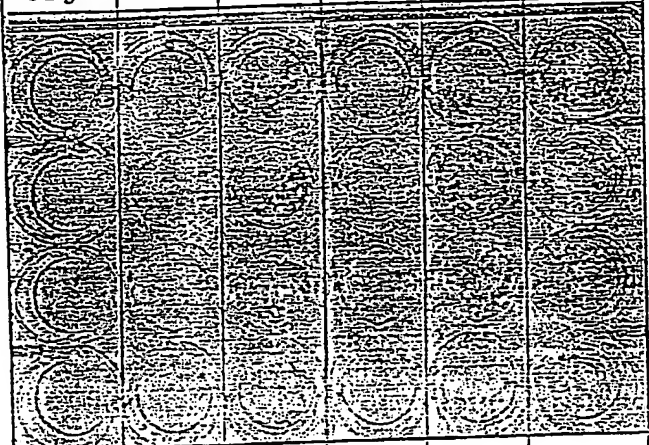


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**FIGURE 2**

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**FIGURE 3**

—	—	3 mg/ml	—	1.5 mg/ml	1.5 mg/ml	Positive Beads
—	3 mg/ml	—	1.5 mg/ml	—	1.5 mg/ml	Negative Beads
3 mg/ml	—	—	1.5 mg/ml	1.5 mg/ml	—	Uncharged Beads
						
---	---	+++	---	+++	+	Size of Aggregates

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FIGURE 4A



FIGURE 4B



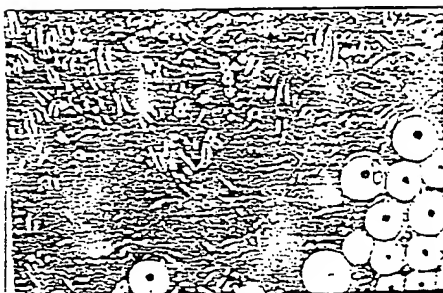
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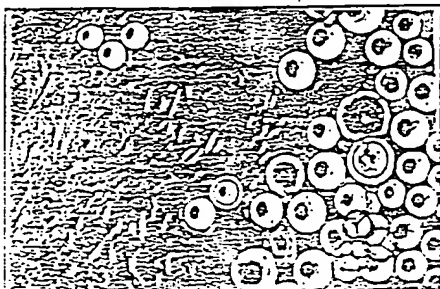
FIGURE 5A

Beads were added 4 hours after  
the cells were added and spread

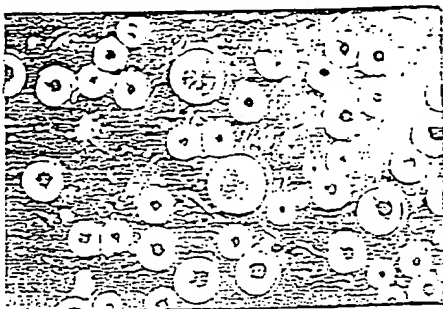
Uncharged  
Beads



Negatively  
Charged  
Beads



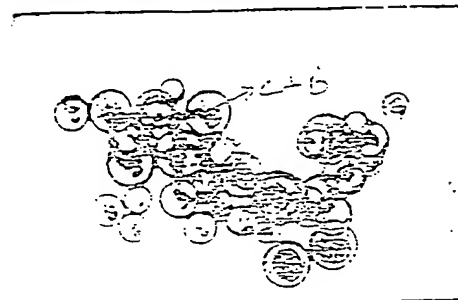
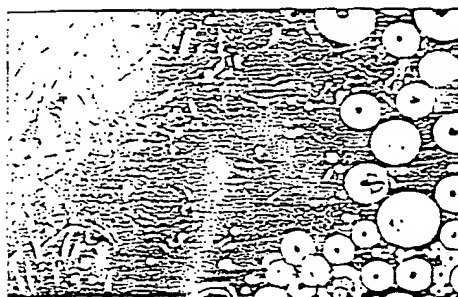
Positively  
Charged  
Beads



C = Cells  
B = Beads

FIGURE 5B

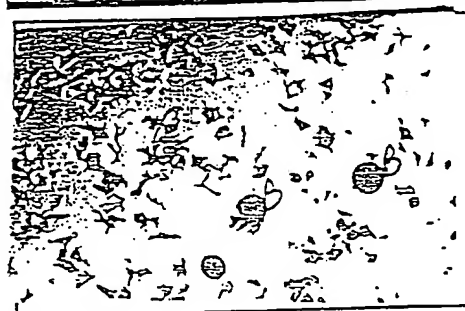
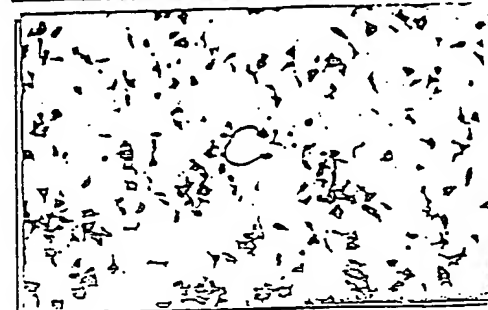
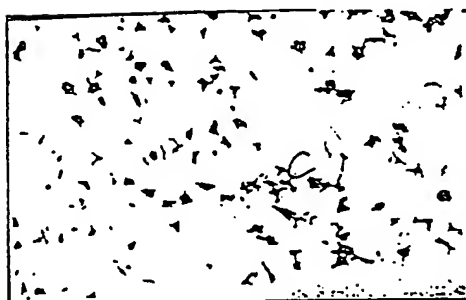
Beads and beads were added at:  
the same time



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FIGURE 6A

Positively Charged Beads

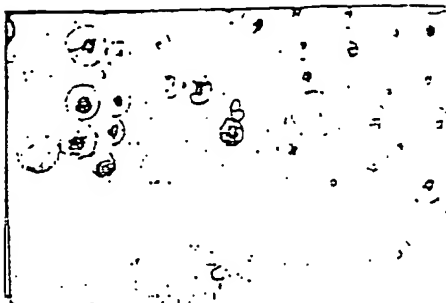
10  
minutes20  
minutes30  
minutesC = Cells  
B = BeadsFIGURE 6BNo  
Beads



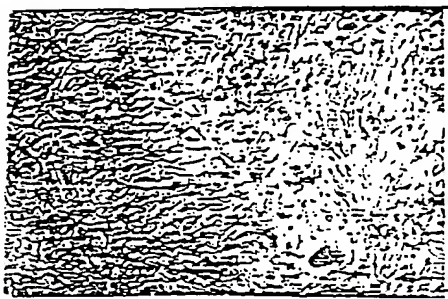
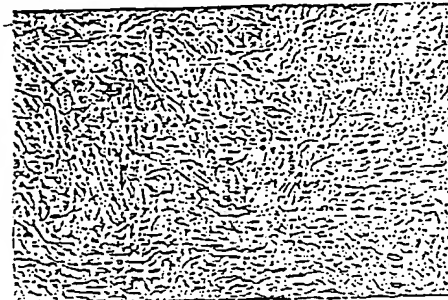
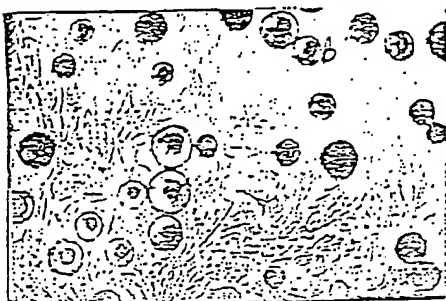
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FIGURE 7AFIGURE 7BPositively Charged  
BeadsUncharged  
Beads

Laminin



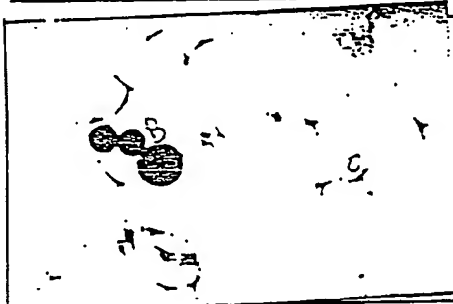
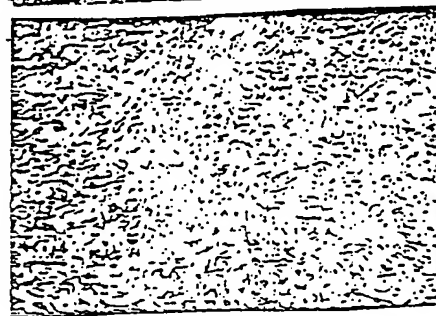
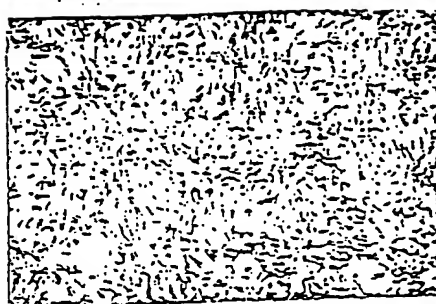
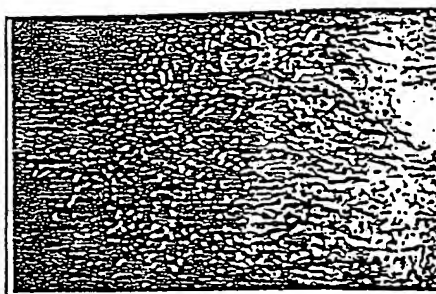
Fibronectin

Collagen  
IVC = Cells  
B = Beads

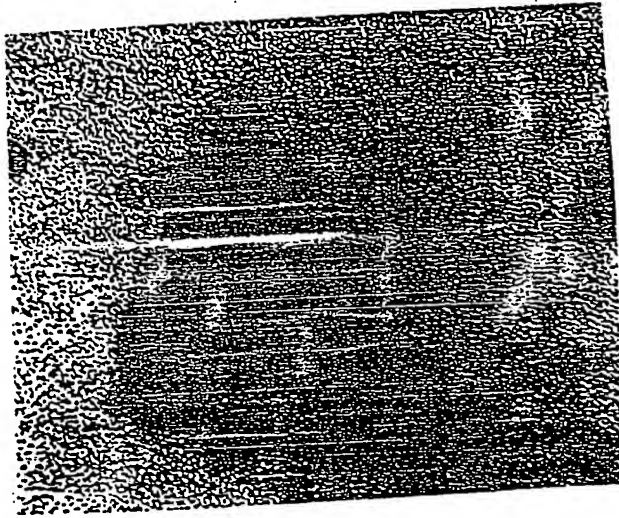
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**FIGURE 8A**Positively Charged  
BeadsPoly-  
lysine

BSA

culture  
dishC = Cells  
B = Beads**FIGURE 8B**Uncharged  
Beads

**FIGURE 9**



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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/05789

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) : C12N 5/00; A01N 43/04, 63/00; A61K 38/00

US CL : 435/325; 424/93.21; 514/44; 530/300

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325; 424/93.21; 514/44; 530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN: MEDLINE, EMBASE, BIOSIS, CAPLUS, CANCERLIT,

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PHILLIPS, T.J. Biologic skin substitutes. J. Dermatol. Surg. Oncol. 1993, Vol. 19; pages 794-800, entire document.	1-5, 19
Y	HUTMACHER et al. A review of material properties of biodegradable and bioresorbable polymers and devices for GTR and GBR applications. International Journal of Oral & Maxillofacial Implants. 05 November 1996, Vol. 11. No. 5, pages 667-678, entire document.	1-25,46-50

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 JULY 2000

Date of mailing of the international search report

25 JUL 2000

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